Feasibility of the determination of polycyclic aromatic hydrocarbons in edible oils *via* unfolded partial least-squares/residual bilinearization and parallel factor analysis of fluorescence excitation emission matrices.

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Abstract

The possibility of simultaneous determining seven concerned heavy polycyclic aromatic hydrocarbons (PAHs) in extra virgin olive and sunflower oils was examined by using unfolded partial least-squares with residual bilinearization (U-PLS/RBL) and parallel factor analysis (PARAFAC), both applied to fluorescence excitation emission matrices. The compounds studied were benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*c,d*]-pyrene, dibenz[*a,h*]anthracene and benzo[*g,h,i*]perylene. The analysis was carried out by fluorescence spectroscopy after microwave assisted liquid-liquid extraction and solid phase extraction on silica. The complexity of the matrix, containing native compounds presenting spectral overlapping with the PAHs and particularly being able to produce inner filter effect, make it necessary the previous sample treatment for a selective detection of PAHs. The U-PLS/RBL algorithm showed the best performance in resolving the heavy PAH mixture in the presence of both the highly complex oil matrix and other unexpected PAHs. The obtained limit of detection with the proposed method ranged from 0.07 to 2 μg kg^{-1}. The U-PLS/RBL predicted concentrations were satisfactorily compared with those obtained by high performance liquid chromatography with fluorescence detection.

Keywords: Polycyclic aromatic hydrocarbons; Edible oils; Fluorescence excitation-emission matrices; Multivariate calibration.

1. **Introduction**

Polycyclic aromatic hydrocarbons (PAHs) constitute a large family of organic compounds containing two or more fused aromatic rings made up of carbon and hydrogen atoms [1]. PAHs stand out mainly by theirs carcinogenic and mutagenic characteristics, especially those of high molecular weight (5-6 fused aromatic rings). These compounds are mostly formed by incomplete combustion of organic matter being continuously released into the atmosphere from natural and anthropogenic sources [2-4].

Human beings are exposed to PAHs contamination mainly by direct inhalation of polluted air or tobacco smoke, direct contact by skin with polluted soils, soot or tars and intake of contaminated water or foods, mainly fatty food (animal or vegetable) [5]. According to Diletti et al. one of the most important source of exposure to PAHs for non smoking humans is food contaminated from air, soil and water and during processing and cooking [6], whereas Barranco et al. propose that the human intake of PAHs from food is considerably higher than that from air or drinking water, edible oils and fats being the most contributing source [7].

The occurrence of PAHs in edible oils is attributed mainly to environmental contamination of vegetable raw material and to contamination coming from some operations carried out during their processing, in which the seed drying, solvent extraction, soil burn, package material and mineral oils used to lubricated the machinery of oil extraction plants represent possible contamination sources [4,8-10].

The carcinogenic and mutagenic characteristics of high molecular weight PAHs justify the careful analytical control of their presence in foods, mainly the fatty one, and make necessary the development of clear cut and uniform legislations. In July 2001, Spain produced a legislation limiting the concentration of eight heavy PAHs in olive pomace oils: benz[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), benzo[*e*]pyrene (BeP), benzo[*a*]pyrene (BaP), indeno[1,2,3-*c,d*]-pyrene (IP), dibenz[*a,h*]anthracene (DBahA), and benzo[*g,h,i*]perylene (BghiP). A maximum limit value of 2 μg kg^{-1} for each single PAH and 5 μg kg^{-1} for the sum of the eight heavy PAHs was established [8,10-12]. In 2003, Chile modified its Sanitary Decree N° 977 of 1996 and established the same maximum limit value of the Spanish legislation for edible oils and fats in general [13].

The two major problems associated with the determination of PAHs in complex matrices, such as vegetable oils and fats, are the diversity of potential interferences present and the low analyte levels [9]. Most methods for PAHs determination usually involve an extraction step, followed by clean-up and finally a chromatographic determination. All of these methodologies are laborious, time consuming and expensive, not only for the sample pretreatment but also for the analytical determination. One alternative to chromatography is fluorescence spectroscopy. Molecular fluorescence measurements can be rapidly and inexpensively performed. Many environmentally important hydrocarbon contaminants are naturally fluorescent and detectable at μg kg^{-1} levels. Unfortunately, the broad nature of fluorescence bands and the large number of fluorescent natural compounds prevent complete analyte selectivity with both excitation and emission based measurements [14,15]. A modern approach to improve the selectivity of this analytical method is the use of advanced chemometric tools, such as second-order multivariate calibration methods. Some second-order methods allow one to directly determine concentrations and estimate spectral profiles of sample components. This property, named the second-order advantage, avoids the physical removal of interferences or the construction of a large and diverse calibration sets [15-19].

The present work explores the possibility of applying the total fluorescence spectroscopy technique, combined with second-order multivariate calibration methods, for the simultaneous determination of seven heavy PAHs in edible oils: BaA, BbF, BkF, BaP, DBahA, BghiP and IP. Microwave assisted liquid-liquid extraction coupled to solid phase extraction with silica was required as a previous sample preparation step. The selected second-order calibration methods were unfolded partial least-squares coupled to residual bilinearization (U-PLS/RBL) and parallel factor analysis (PARAFAC). In addition, some analyses were performed in the presence of nine of the remaining priority EPA (Environmental Protection Agency)-PAHs, as additional potential interferences. Remarkable differences in the prediction capabilities of the employed algorithms are shown and discussed. Finally, the feasibility of determining the seven selected PAHs in edible oils samples is demonstrated.

2. Theory

2.1. Parallel Factor Analysis (PARAFAC).

Excitation-emission fluorescence measurements can provide a three-way data set, in which each sample gives an excitation-emission data matrix (EEM). A series of data matrices obtained for multiple samples make up a three-way array **X**. The PARAFAC algorithm decomposes the data array **X**, and generates a trilinear model that minimizes the sum of the squares of the residuals (e_{ijk}) as indicated in eq (1).

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{if} c_{kf} + e_{ijk} \quad (1)$$

where the element x_{ijk} of **X** represents the datum for sample *i* at the instrumental channels *j* and *k* of (e.g., excitation and emission wavelengths). The three-way data array is thus decomposed into a set of sample scores, a_{if}, loadings for the emission mode, b_{jf}, and loadings for the excitation mode, c_{kf}. The rank of the PARAFAC model is given by the number of factors, *F*, needed to describe the systematic variation in the data array. A crucial stage in the development of the model is the determination of *F* [20-22]. There are various criteria for evaluating *F*, including the percentage of fit and the core consistency test [21, 23], which provide a measure of the variability of the experimental data reflected by the model. Values close to one hundred in both parameters are desirable.

The decomposition of three-way data usually gives a mathematically unique solution for a given number of components. Thus, there are no mathematical ambiguities in the solution except trivial scale and order issues. Therefore, if the PARAFAC model is also a description of the chemically meaningful structure, the parameters of the model will have a chemical interpretation. Specifically, each PARAFAC component will be an estimate of the contribution from one fluorophore, and this estimate is given by a score vector containing the relative concentrations, an emission loading being an estimate of the emission spectrum and an excitation loading being an estimate of the excitation spectrum. Thus, the PARAFAC model can be used for a unique decomposition of the fluorescence data from a complex sample set into a number of PARAFAC components corresponding to the number of fluorophores present in the samples [20]. To evaluate the quality of the retrieved profiles, we used the criterion of similarity (correlation coefficient, *r*), and compared the true spectra with the

spectra obtained from the PARAFAC algorithm. A value of r = 1 indicates total coincidence.

2.2. Unfolded Partial Least Squares (U-PLS).

U-PLS operates in a two-step fashion [24]. First, concentration information is introduced into the calibration step (without including data for the unknown sample), in order to obtain two kinds of latent variables: loading factors contained in matrix **P** and weight loading factors contained in matrix **W**. They are estimated from I calibration data matrices $\mathbf{X}_{c,i}$ (size $J \times K$, where J and K are the number of channels in each data mode) and calibration concentrations **y** ($I \times 1$, where I is the number of calibration samples). The $\mathbf{X}_{c,i}$ matrices are vectorized (unfolded) and grouped into a matrix $\mathbf{Z}_X$ as indicated in eq (2).

$$\mathbf{Z}_X = \left[\mathrm{vec}(\mathbf{X}_{c,1}) \middle| \mathrm{vec}(\mathbf{X}_{c,2}) \middle| \ldots \middle| \mathrm{vec}(\mathbf{X}_{c,I}) \middle| \right] \quad (2)$$

where "vec" denotes the vectorization (unfolding) operation, which converts the $J \times K$ matrices into $JK \times 1$ vectors. With this $\mathbf{Z}_X$ matrix, of size $JK \times I$, a usual PLS model is calibrated [24], furnishing a set of loadings **P** and weight loadings **W** (both of size $JK \times A$, where A is the number of latent factors), as well as regression coefficients **v** (size $A \times 1$). The parameter A is usually selected by techniques such as leave-one-out cross-validation [25]. If the calibration were exact, **v** could be employed to estimate the analyte concentrations in an unknown specimen using the eq (3).

$$y_u = \mathbf{t}_u^T \mathbf{v} \quad (3)$$

where $\mathbf{t}_u$ is the test sample score, obtained by projection of the (unfolded) data for the test sample $\mathbf{X}_u$ onto the space of the A latent factors as indicated in eq (4).

$$\mathbf{t}_u = (\mathbf{W}^T\mathbf{P})^{-1}\mathbf{W}^T\mathrm{vec}(\mathbf{X}_u) \quad (4)$$

2.3. U- PLS with residual bilinearization (U-PLS/RBL).

U-PLS can be coupled to RBL in order to reach the second-order advantage. RBL is a post-calibration procedure, which is based on principal component analysis (PCA) to model the presence of unexpected constituents in a sample [18, 24, 26]. The matrix data $\mathbf{X}_u$ for a sample with unexpected constituents is first vectorized [vec($\mathbf{X}_u$)] and then expressed as shown in eq (5).

$$\text{vec}(\mathbf{X}_u)=\mathbf{P}\mathbf{t}_u+\left[\mathbf{B}_{unx}\mathbf{G}_{unx}(\mathbf{C}_{unx})^T\right]+\mathbf{e}_{RBL} \quad (5)$$

where $\mathbf{e}_{RBL}$ is the residual error RBL term, and $\mathbf{B}_{unx}$, $\mathbf{G}_{unx}$, and $\mathbf{C}_{unx}$ are provided by PCA [which is usually performed by singular value decomposition (SVD)] of a residual matrix, obtained after reshaping the residual vector $\mathbf{e}_{RBL}$ computed assuming that interferences are absent, as indicated in eq (6).

$$\mathbf{B}_{unx}\mathbf{G}_{unx}(\mathbf{C}_{unx})^T=\text{SVD}\{\text{reshape}\,[\text{vec}(\mathbf{X}_u)-\mathbf{P}\mathbf{t}_u]\} \quad (6)$$

where "reshape" indicates the reverse operation of the vectorization, i.e., conversion of a $JK\times1$ vector into a $J\times K$ matrix, and the SVD operation is performed using the first N_{unx} principal components, where N_{unx} indicates the number of unexpected test sample constituents.

The RBL procedure consists in keeping constant the matrix of loadings **P** in eq (5) at the calibration values and varying $\mathbf{t}_u$ in this latter equation in order to minimize the norm of $\mathbf{e}_{RBL}$ ($|\mathbf{e}_{RBL}|$). During the RBL minimization, profiles for the unexpected constituents are continually updated through eq (6). The standard deviation (s_{RBL}) of the residuals in eq (5) can be taken as a measure of the goodness of fit (GOF) for the RBL procedure and according to Bortolato et al. is given by eq (7) [18].

$$s_{RBL}=\|e_{RBL}\|/[(J-N_{unx})(K-N_{unx})-A]^{\frac{1}{2}} \quad (7)$$

Usually, the number of unexpected constituents is estimated by inspection of the behavior of s_{RBL} toward increasing values of N_{unx}. It is assumed that s_{RBL} stabilizes at a value compatible with the instrumental noise when the correct value of N_{unx} is reached. However, when the number of unexpected constituents is large, as in the present case, the latter method does not provide reliable results. This is consistent with the fact that

model selection guided by the GOF generally underestimates the generalization error of a model. An interesting alternative procedure for estimating N_{unx} is to apply the so-called generalized cross-validation (GCV) criterion, which can be adapted to the present case by first defining a penalized residual error (s_{pen}), calculated in an analogous manner to the GCV error:

$$s_{pen} = s_{RBL}\left[(J \times K)/[(J - N_{unx})(K - N_{unx}) - A]^{\frac{1}{2}}\right] \quad (8)$$

To estimate the optimum number of unexpected constituents for RBL, the following ratio was computed for increasing values of N_{unx}:

$$R = s_{pen}(N_{unx})/[s_{RBL}(N_{unx} - 1)] \quad (9)$$

The first value of N_{unx} for which R did not exceed 1 was then selected as the number of RBL components. This is one of the recommended procedures for comparing cross-validation with autoprediction residuals errors [18].

3. Experimental

3.1. Reagents and solutions

Acenaphthylene (ACEN), anthracene (AN), phenanthrene(PHEN), fluoranthene (FLT), fluorene (FLU), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF) and indeno[1,2,3-*c,d*]-pyrene (IP) were purchased from Accustandard (New Haven, CT, USA), acenaphthene (AC), pyrene (PYR) and chrysene (CHR) were obtained from Supelco (Bellefonte, PA, USA), naphthalene (NAPH), benz[*a*]anthracene (BaA) and benzo[*g,h,i*]perylene (BghiP) were purchased from Aldrich (Steinheim, Germany) and benzo[*a*]pyrene (BaP) and dibenz[*a,h*]anthracene (DBahA) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). All reagents were of high-purity grade and used as received.

Solvents acetonitrile, n-hexane and 2-propanol were purchased form Merck (Darmstadt, Germany) and dichloromethane was obtained from Mallinckrodt Chemicals (Phillipsburg, PH, USA). All of them were HPLC grade and used as received.

Stock solutions of pure analytes (100 μg mL^{-1}) were prepared in acetonitrile. From these solutions, more diluted solutions (100 μg L^{-1}) in n-hexane were obtained by taking appropriate volumes, evaporating under a nitrogen stream and diluting with n-hexane. All solutions were stored in silaned amber vials at 4°C in darkness. The solutions were stable for almost six months. PAHs reagents were handled with extreme caution, using gloves and protective clothing.

3.2. Apparatus and software

A Milestone Microwave Laboratory System (Sorisole, BG, Italy) equipped with a high performance microwave digestion unit model mls-1200 Mega, a exhaust module model EM-45/A, a terminal Mega-240 and a 10-position rotor was used for the samples preparation.

A Varian Cary-Eclipse luminescence spectrometer (Mulgrave, Australia) equipped with a xenon flash lamp was used to obtain the excitation-emission fluorescent measurements. A 1.00 cm quartz cells were used and the EEMs were registered in the ranges λ_{exc}: 250-400 nm each 5 nm and λ_{em}: 370-550 nm each 2 nm. The excitation and emission slit widths were 10 nm. The spectra were saved in ASCII format and transferred to a computer for subsequent manipulation.

High performance liquid chromatography with fluorescence detector (HPLC-FLD) analysis was carried out on a liquid chromatograph equipped with a Waters 600 HPLC pump, a fluorescence detector Waters 2475 and auto sampler Waters 717. The column was an Intertsil HPLC ODS-P (250 x 4.6 mm ID, 5 μm particle size) purchased from GL Sciences (Tokyo, Japan). The mobile phase was a mixture of acetronitrile (A) and water (B) at a flow rate of 1.4 mL min^{-1}. A gradient program was used: 0-0.1 min 70% A isocratic; 0.1-10 min linear gradient 90% A; 10-15 min 90% A isocratic; 15-20 min linear gradient 100% A; 20-32 min 100% A isocratic; and finally, back to the initial condition: 32-35 min linear gradient 70% A; 35-38 min 70% A isocratic. An injection volume of 20 μL was employed. Four channels were used to define the excitation and emission wavelengths (λexc/ λem) in the fluorescence detector: channel A 220/330; channel B 292/410; channel C 292/426; and channel D 300/500. Gas chromatography-

mass spectrometry (GC-MS) analysis was performed on a ….marca (ciudad,país) gas chromatograph coupled to a …marca modelo.. mass spectrometer, equipped with vacuum marca, modelo…. and turbomolecular marca modelo pumps. Chromatographic separations was achieved by using a nombre composición……column from…..marca….(ciudad,pais) dimensiones. The samples were analyzed in …..mode, run with a ……source. An injection volume of xxxμL was employed colocar si hubo filtración de la muestra y en que tipo de filtro. Upon positive identification of each specific compound, final quantification was performed using external calibration?.

The routines for data pre-treatment used to eliminate Rayleigh and Raman scatter peaks from EEMs [27] and processing were written in MATLAB [28]. Those employed for PARAFAC and U-PLS/RBL are available on internet [29]. All algorithms were implemented using the graphical interface of the MVC2 toolbox [30], which is also available on internet [31].

3.3. Calibration set samples

A calibration set of 17 samples containing the seven studied PAHs in *n*-hexane was prepared from the diluted solution. Twelve samples of the set corresponded to the concentrations provided by a Plackett-Burman design. The tested concentrations were in the range: 0-2.00 μg L^{-1} for BaA, BbF, BaP and DBahA; 0-0.50 μg L^{-1} for BkF; 0-4.00 μg L^{-1} for BghiP; and 0-2.50 μg L^{-1} for IP. The remaining five samples corresponded to a blank solution, a solution contained all the studied PAHs at an average concentration (1.06 μg L^{-1} for BaA, BbF, BaP, DBahA and BghiP; 0.29 μg L^{-1} for BkF; and 1.50 μg L^{-1} for IP), a solution contained BbF (2.00 μg L^{-1}) and two samples contained BghiP at different concentrations (2.00 and 4.00 μg L^{-1}). The EMMs were then read and subject to second-order data analysis.

3.4. Validation set samples

Eleven samples of an organic EVOO purchased in a local supermarket were used to prepare the validation set. These samples were processed by the sample preparation described bellow and spiked in the final step with different volumes (order of μL) of diluted solutions of the seven selected PAHs. The EMMs were then read and subject to second-order data analysis. The validation sample set was prepared

employing different concentrations from those used for the calibration set and following a random design.

3.5. Test set samples

With the purpose of testing the applicability of the investigated method, the analysis of samples of different brands and kinds of edible oils was performed. To this end four brand of extra-virgin olive oil (EVOO) and two brand of common sunflower oil (SO) samples were purchased at a local supermarket. Because these samples did not contain PAHs or their concentrations were lower than the detection limits of the studied methods, a recovery and predictive capacity study was carried out by spiking them with the seven studied PAHs. Then these samples were processed by the sample preparation described bellow and the EEMs were read. A total of 25 spiked real samples were prepared for this purpose: 17 samples including the four different brands of EVOO and 8 samples including the two brands of SO. These samples were also analyzed by using HPLC-FLD as a reference method [1] (Table 3).

Moreover, another test set of four organic EVOO samples containing the remaining 9 EPA PAHs at 3.00 μg kg^{-1} for each one was prepared in order to probe the predictive capacity of the models in presence of potentially interfering PAHs. The concentrations of the seven heavy studied PAHs were: 4.50 μg kg^{-1} for BaA, BbF, BaP and DBahA; 2.40 μg kg^{-1} for BkF; and 6.00 μg kg^{-1} for BghiP and IP. These latter sets of samples were also analyzed by HPLC-FLD.

Finally a set of thirteen real samples of edible oils (eight brands of EVOO, four brands of SO, and one SO used to prepare fried fish) were analyzed by the propose method and by GC-MS/MS as a confirmatory method.

3.6. Microwave assisted liquid-liquid extraction coupled to solid phase extraction, MAE-SPE.

A glass system previously designed in our laboratory was used in microwave assisted extraction (MAE) [32]. An aliquot of 1.00 ± 0.01 g of oil was accurately weighed into the 50 mL Erlenmeyer flask with ground-glass joint. Then, 30 mL of acetonitrile was added and the air-cooled condenser was adapted to the ground-glass joint of the flask. The glass system was put into the microwave oven and heated for 19

min at 150 W. Only eight of ten positions available in the rotor were used. After cooling, the inner wall of the condenser was rinse with a few milliliters of acetonitrile and removed from the flask. The top layer was carefully transferred with a Pasteur pipette into a 50 mL round-bottom flask and the extract was concentrated to dryness in a vacuum rotary evaporator equipped with a 65 °C water bath. Then the extract was dissolved in 1 mL of n-hexane.

The solid phase extraction clean-up was performed using a 2 g silica SPE cartridge obtained from Supelco (Bellefonte, PA, USA). The 2 g silica cartridge were previously washed with 5 mL of dichloromethane and conditioned with 5 mL of n-hexane. After that, 1 mL of dissolved extract was loaded onto the cartridge (0.5 mL for SO oil) and the PAHs were eluted with 15 mL of n-hexane:dichloromethane 80:20 (v/v) mixture. All the eluate was collected in a 22 mL amber vial and concentrated under nitrogen stream. The residue was dissolved in 3 mL of n-hexane and 2 mL were used to read the EEMs in the luminescence spectrometer. The remaining volume was dried under a nitrogen stream and dissolved in 1 mL of 2-propanol for HPLC-FLD analysis.

4. Results and discussion

4.1. General considerations and sample treatment

Figure 1A and B shows the excitation and emission fluorescence spectra for the seven studied PAHs in n-hexane and the remaining 9 EPA PAHs, respectively. It is clear that overlapping of different degrees occurs among the bands, and the situation becomes more serious if additional PAHs are present. Consequently, their simultaneous fluorimetric determination represents a significant analytical challenge.

Moreover, in a previous work we demonstrated that oil matrices make difficult the PAHs determination due to the presence of pigments (mainly pheophytin and chlorophyll) and tocopherols that produce inner filter phenomena and partial overlapping with the bands of PAHs at short wavelengths, respectively. As a result, even when the second-order methods are used the sample preparation is hard to avoid. The proposed sample preparation combining microwave assisted L-L extraction with SPE on silica permit to concentrate the analytes and eliminate the main interferences for the clear detection of PAHs in edible oils [33].

According to our knowledge and in agree with Moret and Conte [34] no more than 50 mg of oil per gram of silica would be loaded onto the SPE cartridge without observing fat breakthrough in the PAH fraction. For these reason, due to the amount of co-extracted oil in the microwave assisted liquid extraction (88±4 mg and 171±9 mg dissolved in 2 mL as final volume for EVOO and SO, respectively) the volume of loaded on onto SPE cartridge was 1 mL for EVOO samples and 0.5 mL for SO samples.

4.2. Second-order multivariate calibration

Chemometric analysis with PARAFAC and U-PLS/RBL algorithms were applied to the EEM data due to the matrix complexity and the overlapping of PAHs spectra. The best algorithm was defined in the validation step with samples of increasing complexity. Firstly, samples of different brands of EVOO containing the studied analytes were evaluated, then samples of a different kind of edible oil (in this case two brands of SO) spiked with the same PAHs were studied. Finally, samples of organic EVOO containing the 9 remaining EPA PAHs were analyzed.

4.2.1. Validation samples

In order to build the second-order calibration models, EEMs were recorded in a wide spectral range involving the fluorescence signals of all the analytes studied.

PARAFAC was applied to three-way data arrays built by joining the data matrices for each validation sample with those for the set of calibration samples. The selection of the optimum spectral range and the optimum number of factors was performed applying the criterion of similarity (correlation coefficient, *r*), percentage of fit and the core consistency test [21, 23]. In U-PLS/RBL, the selection was made using the cross-validation method described by Haaland and Thomas [25] over the calibration set only. The optimum number of factors is estimated by calculating the ratios $F(A)=\mathrm{PRESS}(A<A^{*})/\mathrm{PRESS}(A)$, where PRESS is the predicted error sum of squares, defined as $\mathrm{PRESS}=\sum_{1}^{I}(y_{\mathrm{nominal}}-y_{\mathrm{predicted}})^{2}$, A is a trial number of factors and A^{*} corresponds to the minimum PRESS. The number of optimum factors was selected as the one leading to a probability of less than 75% and $F > 1$. Notice that RBL is not required for calibration samples, since they carry no unexpected components.

Table 1 shows the number of factors and the final excitation and emission spectral ranges (como se hizo) selected for each analyte when PARAFAC and U-PLS were applied. The optimum spectral ranges for each analyte were almost the same. The number of factors estimated for PARAFAC was equal or lower than that for U-PLS. This is due to the fact that U-PLS provides latent variables (abstract loadings and regression coefficients) that do not have any physical interpretation, and only the adequate fit of the sample signal to the calibration model indicates that the correct analyte is quantified. In contrast, PARAFAC provides physically interpretable profiles, and the identification of the chemical constituents under investigation is done for the comparison of the estimated profiles and those for a standard solution of the analyte of interest.

PARAFAC and U-PLS/RBL were then applied to predict the analyte concentration in the validation samples. In this case, RBL is required for the validation samples since they carry unexpected components due to the oil matrix. Figs. 2A and 2C show the three-dimensional plot of the EEM for a typical calibration sample and an organic EVOO sample respectively. The real challenge we are facing is evident when observing these figures and comparing them with Fig. 2B, which shows the EEM for a typical validation sample that includes the seven studied analytes and the oil matrix. Consequently, when U-PLS/RBL was applied to the validation samples, in addition to the latent variables estimated for each analyte from the calibration set, the introduction of RBL procedure with an additional number o factors corresponding to the unexpected oil constituents was required. The number of RBL factors, estimated by suitable consideration of RBL residues [18], ranged from 1 to 4, depending on the analyzed PAH and the corresponding spectral range.

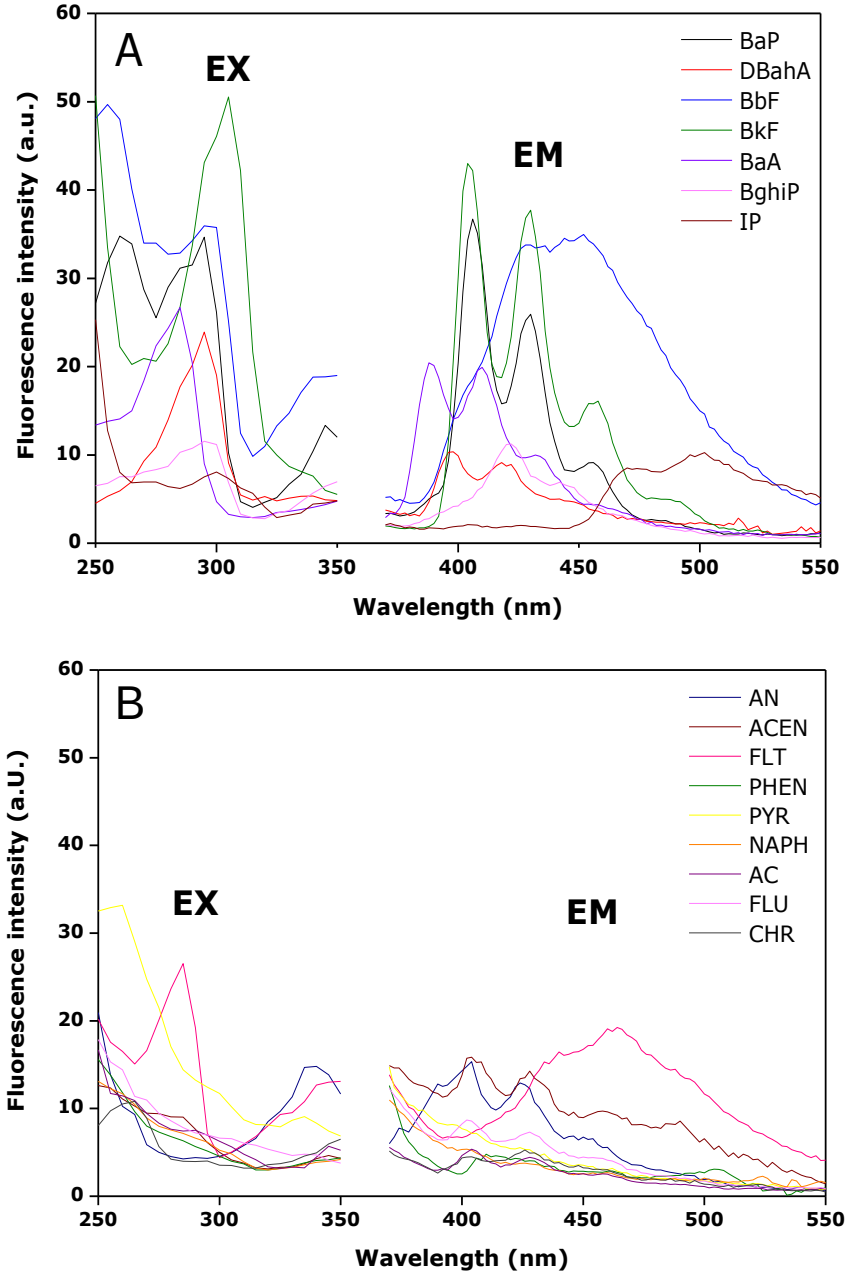

Fig. 1. (A) Excitation (EX) and emission (EM) fluorescence spectra for solutions in n-hexane of 0.50 μg L^{-1} of BkF (green) and 2.00 μg L^{-1} of: BaP (black), DBahA (red), BbF (blue), BaA (violet), BghiP (magenta), and IP (wine), and (B) for AN (blue), ACEN (wine), FLT (pink), PHEN (olive), PYR (yellow), NAPH (orange), AC (purple), FLU (magenta) and CHR (gray). The λex (nm)/ λem (nm) are: 290/410, 270/394, 290/454, 310/406, 290/388, 300/420, and 300/484 for BaP, DBahA, BbF, BkF, BaA, BghiP, and IP respectively and 340/402, 255 386, 290/464, 264/390, 320/394, 275/380, 280/380, 280/380, and 275/380 for AN, ACN, FLT, PHEN, PYR, NAPH, AC, FLU, CHR.

Table 1

Number of factors and excitation-emission ranges used in U-PLS (RBL) and PARAFAC methods.

	PARAFAC			U-PLS and U-PLS/RBL		
	F	Excitation (nm)	Emission (nm)	*A*	Excitation (nm)	Emission (nm)
BaA	6	260-325	374-444	6	260-325	374-444
BbF	5	300-345	376-500	6	300-345	376-500
BkF	4	260-340	370-470	5	260-340	370-450
BaP	6	270-350	378-482	7	270-350	378-482
DBahA	7	260-340	384-462	8	260-340	384-462
BghiP	8	250-330	370-498	8	250-330	370-498
IP	4	320-380	450-490	4	320-380	450-490

The statistical results for the determination of the seven studied PAHs in the validation samples using PARAFAC and U-PLS/RBL are show in Table 2. U-PLS/RBL yields good predictions for PAHs with a relative error (REP) of at most 15%, except in the case of BghiP, with a REP of 25%. However, considering the complexity of the system the latter value can be acceptable. In contrast, a poorer prediction was observed when PARAFAC was applied with REPs above 20%, being BghiP and IP the worst predicted analytes. Actually PARAFAC could not predict the concentrations of BghiP in the validations samples. This fact may be ascribed to the low fluorescence intensity and the significant spectral overlapping of these analytes with the matrix which preclude the successful decomposition of the second-order data.

In Chile and Spain the maximum admissible concentration level for eight heavy PAHs in edible oils is 2 μg kg^{-1} for each single PAH, including BaA, BbF, BkF, BaP, BeP, DBahA, BghiP, and IP, and 5 μg kg^{-1} for the total PAH content. The limits of detection (LODs) obtained with U-PLS/RBL were bellow 1 μg kg^{-1}, except for BghiP and IP (1.8 and 2.0 μg kg^{-1}, respectively). Although the LOD for these analytes are in the order of 2.0 μg kg^{-1}, it can be acceptable for its determination in potentially contaminated samples. Moreover, the LODs obtained by U-PLS/RBL are in the order of those reported for HPLC-FLD methods [2, 4, 7, 9, 35]. Although LODs obtained with

PARAFAC were bellow 2.0 μg kg^{-1} for BaA, BbF, BkF and DBahA, they were almost 2 or 3 times higher than those obtained by U-PLS/RBL. The highest LODs were obtained for BaP and IP (4.6 and 4.8 μg kg^{-1} respectively) and exceeded the established limits for each single PAH.

The poor results obtained with PARAFAC could be attributed to the inability to model a system where the spectroscopic profiles of the analytes are similar among them and also with the matrix component. Thus only U-PLS/RBL was applied for the prediction of PAHs in the rest of the edible oil samples.

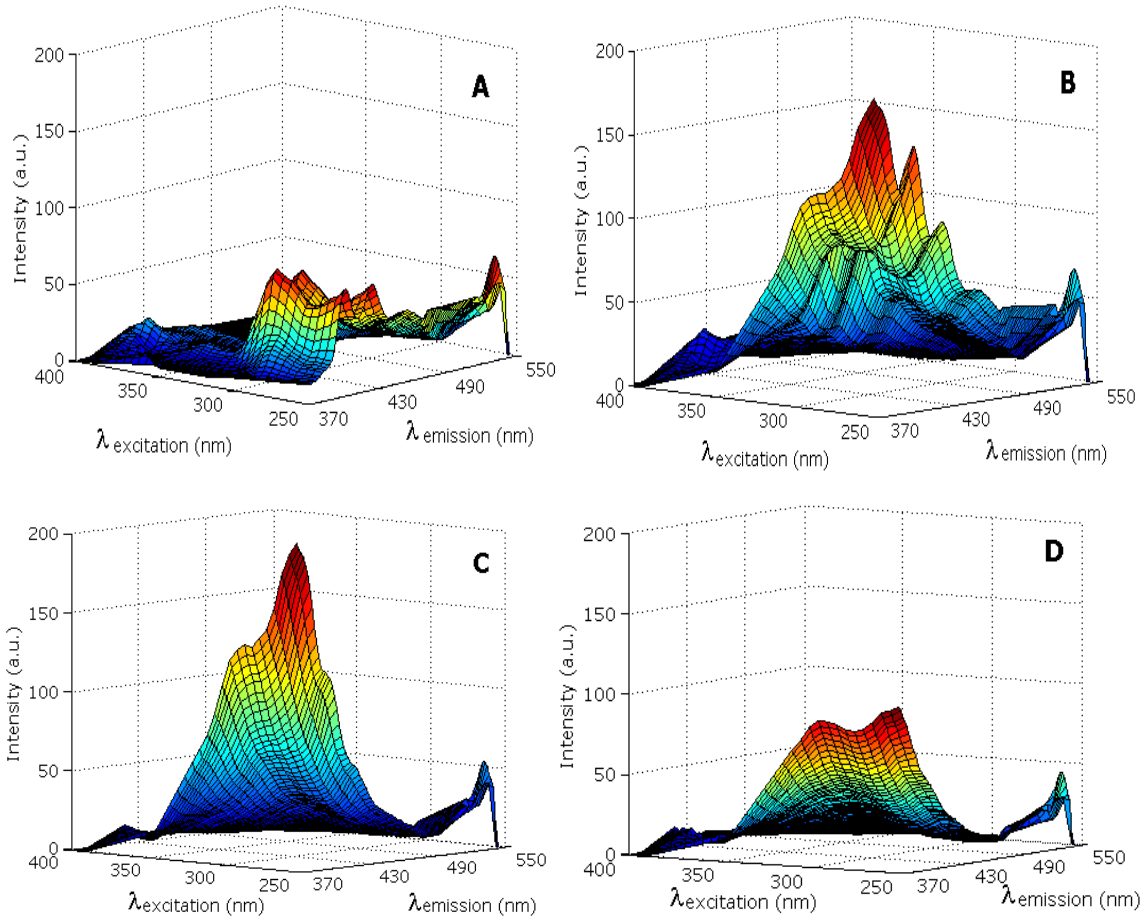

Fig. 2. Three-dimensional plots for excitation-emission fluorescence matrices corresponding to A a calibration sample containing 1.06 μg L^{-1} of BaP, DBahA, BbF, BaA and BghiP, 0.29 μg L^{-1} BkF, and 1.50 μg L^{-1} of IP, B a validation sample containing the seven PAHs at the same concentrations of the calibration sample, C an organic EVOO sample, and D a SO sample.

Table 2

Statistical results for the determination of PAH in validation samples.

	PARAFAC							U-PLS/RBL						
	BaA	BbF	BkF	BaP	DBahA	BghiP	IP	BaA	BbF	BkF	BaP	DBahA	BghiP	IP
RMSEP (μg kg^{-1})[a]	0.62	1.2	0.25	1.1	0.59	-	3.4	0.24	0.43	0.09	0.35	0.26	0.89	0.52
REP (%)[b]	25	42	37	46	24	-	98	10	15	13	15	11	25	15
γ^{-1} (μg kg^{-1})[c]	0.27	0.34	0.07	1.4	0.38	-	1.5	0.16	0.13	0.02	0.11	0.16	0.54	0.61
LOD (μg kg^{-1})[d]	0.91	1.1	0.24	4.6	1.3	-	4.8	0.51	0.43	0.07	0.35	0.53	1.8	2.0

[a] Root mean square error of prediction, $RMSEP=[(1/I)\sum_1^I (c_{nominal}-c_{predicted})^2]^{1/2}$ where I is the number of prediction samples and $c_{nominal}$ and $c_{predicted}$ are the actual and predicted concentrations, respectively.

[b] Relative error of prediction, $REP=100*RMSEP/\bar{c}$ where $\bar{c}$ is the mean calibration concentration.

[c] Inverse of the analytical sensitivity (γ), $\gamma^{-1}=s_x/SEN_n$ where s_x is the instrumental noise and SEN_n is the sensitivity. The s_x and SEN_n values are averages of the values corresponding to eleven validation samples.

[d] Limit of detection, $LOD=3.3*\gamma^{-1}$, reference [36].

4.2.2 Test samples of spiked edible oils.

The predictive capacity of the calibration model using U-PLS/RBL was evaluated with different brands and kinds of edible oils. A total of 25 spiked samples of edible oils were analyzed: 17 samples including four different brands of EVOO and 8 samples including two brands of SO. Figures 2C and 2D shows the three-dimensional plot of the EEM for an organic EVOO and an SO sample respectively. In spite of the apparent fluorescence intensity differences between the EVOO an SO samples, the excitation-emission profiles are similar. Consequently, additional RBL factors than the estimated for the validation samples were not required for the predictions of the analytes with U-PLS/RBL. Table 3 shows the mean recovery and predictive capacity results obtained for the determination of the seven studied PAHs in these samples.

As can be observed, the recoveries obtained with U-PLS/RBL ranged 64-81% and were in agreement with the values previously reported for the preparation sample method employed in this study [33]. On the other hand, no significant differences between predicted concentrations by U-PLS/RBL and obtained concentrations for HPLC-FLD were observed (Table 3), consequently the theoretical (1,0) point are included in the ellipses or close to the border. Figure 3 shows the plots of the U-PLS/RBL predicted concentrations as a function of the obtained values by HPLC-FLD

and the corresponding elliptical joint regions (at 95% confidence level) for BaA and BaP (representative compounds of the studied group), respectively. In addition, the mean recoveries (in percentage) for the 25 samples were calculated and compared by a paired *t*-test and no significant differences between the methods were observed (*p*-value: 0.80 at 95% confidence). Therefore, on the basis of the obtained results, the proposed method using EEMs coupled with U-PLS/RBL is comparable with the reference HPLC-FLD method.

Table 3 Part A

Recovery and predictive capacity study for seven studied PAHs in spiked samples of different kinds and brands of edible oil samples using U-PLS/RBL and HPLC-FLD as a reference method.

			BaA			BbF			BkF			BaP		
Oil	Brand	Sample	Nominal (μg kg^{-1})	U-PLS/RBL Predicted (μg kg^{-1})	HPLC-FLD (μg kg^{-1})	Nominal (μg kg^{-1})	U-PLS/RBL Predicted (μg kg^{-1})	HPLC-FLD (μg kg^{-1})	Nominal (μg kg^{-1})	U-PLS/RBL Predicted (μg kg^{-1})	HPLC-FLD (μg kg^{-1})	Nominal (μg kg^{-1})	U-PLS/RBL Predicted (μg kg^{-1})	HPLC-FLD (μg kg^{-1})
EVOO	A	1	5.4	3.1	3.4	4.8	4.2	3.0	1.2	1.0	0.85	4.5	1.7	2.3
		2	0.60	<LOD	0.56	5.7	3.7	3.7	0.60	0.27	0.31	4.8	2.5	2.7
		3	5.4	3.6	3.9	3.9	2.8	2.9	1.2	0.79	0.89	1.2	0.83	0.79
		4	3.9	2.8	3.1	5.1	3.4	3.8	0.30	0.28	0.27	3.0	1.8	2.0
	B	5	0.60	0.87	0.67	5.7	4.2	4.0	0.40	0.38	0.36	2.7	1.8	1.6
		6	1.5	1.7	1.4	3.9	3.4	3.1	1.2	1.3	0.97	3.9	2.9	2.5
		7	3.0	2.5	2.5	4.5	3.4	3.3	0.90	0.80	0.63	4.2	2.9	2.4
		8	5.7	4.1	5.2	2.4	2.0	2.1	0.90	0.70	0.76	4.5	3.1	3.2
	C	9	5.7	4.2	4.8	0.9	1.0	0.80	1.2	0.98	1.1	1.5	0.85	1.3
		10	0.90	0.7	0.76	4.2	2.9	3.0	0.60	0.34	0.45	3.9	2.3	2.6
		11	5.7	4.2	4.5	1.5	1.6	1.3	0.60	0.52	0.53	3.9	2.3	2.6
		12	5.7	4.3	4.3	0.6	0.68	0.59	1.8	1.3	1.4	0.90	0.41	0.68
	D	13	2.7	1.9	2.2	4.8	3.7	3.6	0.2	0.22	0.28	0.60	0.84	0.56
		14	4.8	3.8	3.7	1.8	2.1	1.4	0.9	0.66	0.70	3.0	2.3	2.1
		15	0.90	0.96	1.0	5.7	4.5	4.9	1.5	1.1	1.3	5.7	4.2	4.0
		16	2.4	2.0	2.0	2.4	2.0	2.0	0.3	0.28	0.31	2.1	1.4	1.5
		17	5.4	3.4	3.9	2.1	1.4	1.6	0.6	0.29	0.45	3.6	2.2	2.1
SO	E	18	6.0	3.3	4.6	11	7.5	7.6	2.4	1.5	1.8	9.6	5.8	5.9
		19	11	6.7	7.3	6.0	4.3	3.7	1.8	1.2	1.4	9.0	5.5	5.9
		20	11	6.0	7.4	9.0	6.1	6.2	1.2	0.72	0.90	7.2	4.6	4.5
		21	7.8	5.0	5.6	10	6.1	6.2	0.6	0.42	0.54	6.0	3.4	3.1
	F	22	6.0	7.2	6.7	11	8.4	10	2.4	1.4	1.6	9.6	6.0	7.2
		23	11	7.1	7.8	6.0	4.6	4.8	1.8	1.2	1.7	9.0	5.3	6.1
		24	11	9.5	7.3	9.0	7.1	6.4	1.2	0.93	1.1	7.2	4.7	4.7
		25	7.8	5.4	5.9	10	7.0	7.1	0.6	0.54	0.60	6.0	3.9	4.0
Mean Recovery (n=25)				78	82		80	76		76	83		65	66

Table 3 Part B

Recovery and predictive capacity study for seven studied PAHs in spiked samples of different kinds and brands of edible oil samples using U-PLS/RBL and HPLC-FLD as a reference method.

			DBahA			BghiP			IP		
Oil	Brand	Sample	Nominal ($\mu g\ kg^{-1}$)	U-PLS/RBL Predicted ($\mu g\ kg^{-1}$)	HPLC-FLD ($\mu g\ kg^{-1}$)	Nominal ($\mu g\ kg^{-1}$)	U-PLS/RBL Predicted ($\mu g\ kg^{-1}$)	HPLC-FLD ($\mu g\ kg^{-1}$)	Nominal ($\mu g\ kg^{-1}$)	U-PLS/RBL Predicted ($\mu g\ kg^{-1}$)	HPLC-FLD ($\mu g\ kg^{-1}$)
EVOO	A	1	1.2	0.95	0.75	1.2	<LOD	0.87	2.4	0.8	1.6
		2	4.5	3.0	2.2	7.5	4.3	3.7	5.7	4.0	3.3
		3	1.5	2.1	1.1	5.7	5.4	3.3	2.1	2.5	1.7
		4	3.0	2.5	2.0	4.2	3.0	2.6	3.9	1.9	2.4
	B	5	4.2	3.0	2.6	9.9	4.0	5.6	1.8	<LOD	1.7
		6	5.4	4.4	3.6	6.9	3.6	4.3	4.5	4.2	3.3
		7	5.7	3.0	3.6	6.3	4.0	3.6	1.8	<LOD	1.4
		8	3.0	3.3	2.4	11	6.1	6.3	4.8	4.1	3.8
	C	9	0.90	1.0	0.64	3.3	2.4	2.4	5.1	4.8	3.9
		10	0.90	1.2	0.64	9.0	6.0	5.0	5.7	3.5	3.9
		11	1.5	1.4	1.1	9.0	6.2	5.3	3.3	2.4	2.3
		12	5.1	3.1	3.4	4.5	4.3	2.4	6.9	5.4	4.7
	D	13	1.5	1.4	1.2	6.9	5.0	4.2	6.0	2.8	4.3
		14	4.8	3.6	3.4	0.9	<LOD	0.64	3.9	3.0	2.9
		15	1.2	1.2	1.2	0.6	<LOD	0.65	7.5	5.2	5.3
		16	5.4	3.8	4.0	1.5	<LOD	1.2	2.7	1.2	1.9
		17	2.1	1.4	1.5	6.6	1.9	3.6	7.2	3.2	4.6
SO	E	18	9.0	5.9	5.9	15	8.8	8.9	11	6.8	7.4
		19	6.0	3.2	4.6	22	13	16	13	6.8	8.9
		20	7.8	4.7	5.5	17	9.4	11	14	8.2	9.5
		21	7.2	4.4	4.9	23	12	13	12	5.5	7.7
	F	22	9.0	6.6	7.0	15	11	13	11	6.5	11
		23	6.0	5.8	4.9	22	13	15	13	8.6	9.4
		24	7.8	5.2	5.5	17	13	11	14	8.3	9.4
		25	7.2	5.1	5.3	23	12	15	12	5.7	8.3
Mean Recovery (n=25)				81	71		64	65		66	71

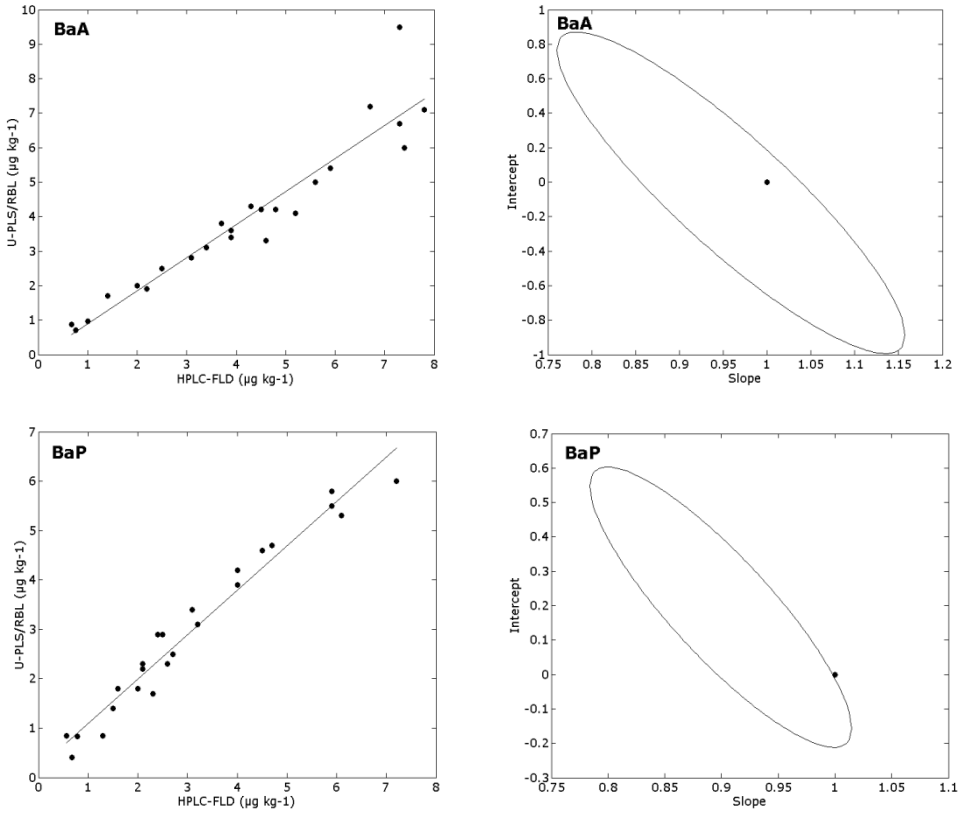

Fig. 3. Plots for U-PLS predicted concentrations as a function of the HPLC-FLD obtained values for BaA and BaP and the corresponding elliptical joint regions (at 95% confidence level) for the slopes and intercepts of the regression for U-PLS/RBL versus HPLC-FLD plots. Black circle in the elliptical plots mark the theoretical point (intercept: 0, slope: 1).

4.2.3 Test samples containing unexpected PAHs

With the purpose of evaluating the capacity of U-PLS/RBL to resolving the seven PAHs selected in presence of the remaining nine EPA-PAHs priority pollutants, four samples of organic EVOO spiked with the studied PAHs and AN, ACN, FLT, PHEN, PYR, NAPH, AC, FLU, and CHR (as a potential interferences), were processed. The interferences were analyzed at 3 µg kg^{-1} of each one; all samples were spiked at the same level of concentration for the heavy PAHs. When U-PLS/RBL was applied to these test samples no additional RBL factors than the used for the validation samples were required for the interferences. This could be ascribed to the fact that these PAHs have fluorescence profiles which do not significantly overlap with those of the studied compounds. Table 4 shows the prediction results corresponding to the application of U-

PLS/RBL and the values obtained by HPLC-FLD. The comparison between the methods was carried out for each PAH by applying a mean-t test to the set of evaluated organic EVOO samples [37]. The null hypothesis corresponds to the null difference between both predictions. The *t* values obtained for $n_1 + n_2 - 2$ degrees of freedom (where n_1 and n_2 are the number of samples processed by U-PLS/RBL and HPLC respectively) at 98% significance, compare favorably with the corresponding tabulated value ($t_{crit(0.02,2)} = 3.14$) for all studied PAHs, suggesting that the values for each PAH concentrations obtained by applying the proposed method are statistically comparable with those provided by the reference HPLC-FLD technique. On the other hand this result suggest that the remaining nine EPA-PAHs which may be possibly present in the edible oils don not produce a significant interference in the proposed analysis.

4.2.4 Real samples

Nos falta confirmación por GC-MS/MS

5. Conclusions

Fluorescence excitation-emission matrices associated to U-PLS/RBL has been demonstrated to be a powerful tool to resolve a mixture of heavy PAHs, in the presence of a very complex matrix as edible oil. Even though the method can resolve the mixture of analytes in the presence of unexpected compounds, the complexity of the matrix with native compounds presenting spectral overlapping with the PAHs and particularly being able to produce inner filter effect, make a sample treatment necessary for a selective detection of PAHs. Thus, the combination of microwave assisted L-L extraction with SPE on silica has permitted to concentrate the analytes and eliminate the interferences. The strength of the proposed method was tested by predicting the heavy PAHs into different brands and kind of edible oils, and in the presence of the remaining EPA-PAHs priority pollutants. The U-PLS/RBL predicted concentrations were compared with the values obtained for HPLC-FLD and no significant differences between them were observed. Moreover, it should be noted that the LOD obtained with the proposed method are still comparable to those reported via HPLC methods. Hence, the proposed method using EEMs coupled with U-PLS/RBL is comparable and a suitable alternative to chromatographic method.

Table 4 Part A

Determination of the seven studied PAHs concentrations in presence of the remaining nine EPA-PAHs priority pollutants[a] in spiked organic EVOO samples using UPLS/RBL and HPLC-FLD as a reference method.

	BaA			BbF			BkF			BaP		
	U-PLS/RBL ($\mu g\ kg^{-1}$)	HPLC-FLD ($\mu g\ kg^{-1}$)	t^b	U-PLS/RBL ($\mu g\ kg^{-1}$)	HPLC-FLD ($\mu g\ kg^{-1}$)	t^b	U-PLS/RBL ($\mu g\ kg^{-1}$)	HPLC-FLD ($\mu g\ kg^{-1}$)	t^b	UPLS-RBL ($\mu g\ kg^{-1}$)	HPLC-FLD ($\mu g\ kg^{-1}$)	t^b
Sample 1	3.0	3.2		2.9	2.6		1.5	1.5		2.4	2.2	
Sample 2	3.8	3.5		3.3	3.0		1.6	1.7		2.6	2.6	
Sample 3	2.8	3.5		3.3	2.8		1.9	1.7		2.9	2.5	
Sample 4	2.5	3.5		3.3	2.9		1.8	1.7		2.6	2.6	
Mean concentration (n=4)	3.0	3.4	1.39	3.2	2.8	2.85	1.7	1.7	0.48	2.6	2.5	1.07

[a] Concentration of the remaining nine EPA-PAHs= 3 $\mu g\ kg^{-1}$

[b] Calculated student t for a mean-t test. The critical t value for $(n_1 + n_2 - 2)$ degrees of freedom and at 98% significance level is $t_{crit(0.02,2)} = 3.14$ [37].

Table 4 Part A

Determination of the seven studied PAHs concentrations in presence of the remaining nine EPA-PAHs priority pollutants[a] in spiked organic EVOO samples using U-PLS/RBL and HPLC-FLD as a reference method.

	DBahA			BghiP			IP		
	U-PLS/RBL (μg kg^{-1})	HPLC-FLD (μg kg^{-1})	t[b]	U-PLS/RBL (μg kg^{-1})	HPLC-FLD (μg kg^{-1})	t[b]	U-PLS/RBL (μg kg^{-1})	HPLC-FLD (μg kg^{-1})	t[b]
Sample 1	2.7	2.6		2.9	2.8		3.4	3.5	
Sample 2	2.5	2.9		2.9	3.3		3.9	3.8	
Sample 3	3.1	2.8		2.7	3.2		5.4	3.8	
Sample 4	2.8	2.8		1.7	3.3		4.7	4.0	
Mean concentration (n=4)	2.8	2.8	0.00	2.6	3.2	1.93	4.4	3.8	1.27

[a] Concentration of the remaining nine EPA-PAHs= 3 μg kg^{-1}

[b] Calculated student t for a mean-t test. The critical t value for (n_1 + n_2 -2) degrees of freedom and at 98% significance level is $t_{crit(0.02,2)}$ = 3.14 [37].

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